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(54) Title: ANTIGEN–PRESENTING CELLS AND THEIR USE IN THERAPY

(57) Abstract

A method for *ex vivo* treatment of antigen presenting cells, wherein said method consists of *in vitro* culture of antigen presenting cells under conditions resulting in development of tolerogenic antigen presenting cells, such tolerogenic cells being characterised by: a) reduced induction of T cell activation upon T–cell receptor ligation as can be determined by any of the measurements selected from the group measurement of proliferation, measurement of cytokine production, measurement of cytotoxicity and measurement of expression of activation cell surface markers, b) a dominant tolerogenic effect. Also part of the invention are such tolerogenic antigen–presenting cells and artificial analogues, compositions containing them and their use in immunotherapy.

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ANTIGEN-PRESENTING CELLS AND THEIR USE IN THERAPY

Field of the invention

[0001] The present invention is in the field of immunotherapy. The invention relates to a novel type of antigen presenting cells, capable of down-regulating T cell responses, and thus suitable for use in the treatment of autoimmune diseases, allergy and transpantation.

Introduction

[0002] For the generation of a specific immune response, the antigen-presenting cell (APC) plays a central role by recruiting and interacting with Ag-specific CD4+ and CD8+ T cells, leading to activation of all three cell-types involved (1-4). The stimulatory capacity of APC can be modulated through signals from the micro-environment, such as cellular interactions (e.g. CD40/CD40L) or soluble factors (e.g. IL-12, IL-10), which could lead to alterations in the cytokine production by APC, as well as in its ability to induce proliferation or cytokine production in T cells (2-6). APC could also be crucial participants in the maintenance and re-establishment of peripheral tolerance through the formation of immunoregulatory clusters, in which the APC serves to bring together regulatory T cells and T cells that are to be regulated (7). Immunotherapy aiming at the specific modulation of APC function could therefore be a novel approach for intervention in T cell-mediated disease processes.

[0003] Regulatory T cells are part of the normal peripheral T lymphocyte pool (8, 9). They can function by suppressing aberrant immune reactivity, e.g. in the case of autoimmunity, or by terminating an immune response to prevent chronic inflammation. Several T cell subsets have been proposed to mediate protective immunoregulatory effects, including Th2 cells (10), IL-10 or TGFβ producing cells (11, 12), $CD45RC^{low}$ cells (13) and CD4+CD25+ T cells (14-16). Recently, it has been shown that also anergic T cells can function as regulatory T cells by suppressing the responses of other T cells (17-19). In a previous paper of our group we described that anergic T cells exerted their suppressive effects in an active and Ag-specific manner, which was dependent on cell-cell contact between anergic T cells and APC, and was not mediated via soluble factors (19).

[0004] T cell anergy has been proposed to be one of the mechanisms to maintain peripheral tolerance (20), and can be defined as a cellular state in which a T cell is alive but fails to proliferate and produce IL-2 upon antigenic restimulation under otherwise stimulatory conditions (21). However, although in general anergy has been regarded as a way to functionally inactivate e.g. autoreactive T cells, based on the recent findings it should be considered that anergic T cells can be involved as active regulators of the immune response as well (19). A way of anergy induction which could operate under physiological circumstances is T-T presentation. Activated human and rat T cells express major histocompatibility complex (MHC) class II molecules on their surface, and can therefore present Ag to other T cells (22, 23). It was shown for both human and rat T cells that Ag presentation by T cells resulted in T cell anergy rather than T cell activation upon antigenic restimulation (17, 19, 24).

Summary of the invention.

[0005] It is found that rat CD4+ T cells which are rendered anergic through T-T presentation, down-regulate the T cell-activating capacity of the APC in an Ag-specific manner. Upon removal of the anergic T cells, the tolerogenic phenotype of APC persisted, indicating that anergic T cells

conditioned the APC to become a mediator of T cell suppression. Importantly, anergic T cells were able to turn previously activated APC into tolerogenic APC. Thus the exact tuning of the APC by anergic T cells is supposed to be a key event in the regulation of the immune response.

[0006] The invention thus pertains, in a first aspect, to isolated tolerogenic antigen-presenting cells (APC). The tolerogenicity of these APC can be induced by treatment of an APC with anergic T cells or active components or analogues thereof under tolerogenicity-inducing conditions, and the tolerogenicity is characterised by a) reduced induction of T cell activation upon T-cell receptor ligation and by b) a dominant tolerogenic effect. The reduced induction of T cell activation can be determined by measurement of proliferation, measurement of cytokine production, measurement of cytotoxicity and measurement of expression of activation cell surface markers. The reduced induction is such that the T-cell response is inhibited by the resulting tolerogenic antigen-presenting cell by at least 50%. The treatment of the APC with anergic T cells or components or analogues thereof typically proceeds in the presence of their specific peptide/MHC complex (autoantigen, allergen) on the APC. The active components or analogues to be used for rendering the APC tolerogenic, are derived from anergic T cells or mimick the component or action of the component(s) on the antigen presenting cell that is or are responsible for the development of tolerogenicity. The tolerogenicity is maintained after separating the antigen presenting cells from the anergic T cells or the components to induce tolerogenicity. The APC are especially professional APC, i.e. B cells, macrophages and dendrites and combinations thereof, especially B cells or macrophages.

[0007] In a second aspect, the invention pertains to a pharmaceutical composition comprising a tolerogenic antigen-presenting cell as defined above in combination with a pharmaceutical carrier. The composition can be in any medicinal dosage form, in particular formulations for subcutaneous and intravenous injection. Tolerogenic APC can be dispensed in isotonic solutions suitable for subcutaneous and intravenous injection. In total 0.5 million to 50 million tolerogenic APC per kilo body weight will be injected to achieve a therapeutic benefit as determined by standard clinical procedures.

[0008] Advantageously, the composition also comprises a therapeutic peptide or protein, in particular an auto-antigen or an allergen. Suitable examples thereof are myelin basic protein (MBP), myelin oligodendrocyte-associated protein (MOG), collagen, heat-shock proteins, Der P1, Fel D1, acetylcholine receptor molecules. The composition may also comprise a minor and major histocompatibility complex (MHC) peptide or protein (see example 3). The heat-shock proteins are especially mycobacterial hsp65, hsp 60 (GroEL), DnaJ, hsp70 family (DnaK), ubiquitin, hsp10 (GroES), low molecular weight hsp's (20-30 kDa), hsp47, hsp56, TCP-1 (T complex peptide), hsp90, hsp104/110, or the fragments of 7-50 amino acids of such heat shock proteins as selected according to the method of WO 95/25744, which is herein incorporated by referefence.

[0009] The therapeutic peptide or peptide may also be a peptide or protein suitable for the prophylaxis or treatment of arthritis, as described in PCT/NL99/00189, incorporated herein by reference. Such peptides or proteins comprise a contiguous sequence of 9 amino acid residues X1-X2-X3-X4-X5-X6-X7-X8-X9, wherein X2, X6 and X7 are any amino acid, X1 is any amino acid except K,H,R,E,D, X3 is S or T, X4 is one of F,L,I,V,A,G,C,P, X5 is one of A,G,C,P,S,N,T,V, X8 is one of V,L,I,M, and X9 is E or D. Such a peptide typically has 9-50 aminoacids, and can be derived from a mammalian cartilage, joint or arthritis-related protein such as collagen or a cartilage protein, and be

capable of being recognised by freshly isolated A2b-like T cells from rats in which Adjuvant Arthritis is induced and/or be capable of being recognised in vitro by T cell clone A2b.

[0010] The addition of a therapeutic peptide or protein is especially useful in an alternative mode of treatment. According to this alternative mode, APC from the patient's blood can be made tolerogenic with T cells which are not derived from the patient (e.g. a T cell line) and which were made anergic by incubation with an antigen not related to the disease to be treated, e.g. an influenza antigen. The APC thus rendered aspecifically tolerogenic can then be administered together with an antigen related to the disease to be treated, e.g. a cartilage peptide in case of a rheumatoid disease.

[0011] Therapeutic antigens can be added at a concentration ranging from 0.1 to 100 microgram per milliliter to the culture medium containing autologous APC and anergic T cells during an overnight incubation step. The next day excess (non-bound) therapeutic antigen can be removed by applying multiple washing steps. After injection of tolerogenic APCs loaded with therapeutic antigen, disease-specific T cells reactive with the therapeutic antigen will be down-regulated in vivo and as a result disease activity will be reduced.

[0012] The pharmaceutical composition of the invention may also contain the tolerogenic APC in artificial form, e.g. in the form of a liposome, to which the functionalities of the APC are bound, such that it is capable of effecting the reduced T-cell induction. MHC-containing-liposomes can be produced, e.g. from egg-yolk phosphatidylcholine, phosphatidylglycerol, cholesterol and purified MHC molecules through detergent removal. Relevant peptides are loaded on the incorporated MHC molecules. Specific components needed for the functionality of the tolerogenic aritifcial APC can be incorporated into the lipid bilayer or included in the liposomes. Such specific components can be identified by determining the difference in expression profile of cytokines and cell surface proteins between non-tolerised APC and tolerogenic APC. The cytokines and/or cell surface proteins and/or other soluble factors associated with the tolerogenic APC and not with the non tolerised APC are then bound to the liposome. Liposome particle sizes may range from e.g. 50 to 250 nm. The tolerogenic liposomes can be administered in amounts corresponding to those for the APC described above.

[0013] In a still further aspect, the invention pertains to a method for inducing T-cell tolerance, which method comprises administering to a subject requiring such induction a therapeutically effective amount of a tolerogenic APC as defined above or a pharmaceutical composition containing it. Such induction may be required in the prophylaxis and/or treatment of auto-immune diseases, including rheumatoid arthritis, multiple sclerosis, psoriasis and diabetes, or for alleviating allograft rejection phenomena or for the treatment of allergies. The dosages to be administered can be determined by the skilled immunotherapist taking into account the nature and level of the diseased state, and the status of the subject in need of treatment.

[0014] The invention furthermore relates to a method for determining the identity of and subsequently optionally isolating or producing a factor or factors responsible for the inhibition of antigen specific T-cell response. Such method may require determining the difference in expression of cytokine and cell surface proteins between APC that are normal i.e. have not undergone the tolerogenicity-inducing treatment defined above, and tolerogenic APC. Alternatively, nucleic acid sequences encoding such factors can be identified and produced, by determining the difference in mRNA expression of cytokine between such normal and tolerogenic APC. Established technologies to determine differential gene expression should be applied. Such technologies include differential

PCR, substractive cDNA library and gene array technology known to individuals skilled in the art.

Detailed description of the invention

[0015] It was found according to the invention that anergic T cells down-regulate the T cell-activating capacity of APC. APC down-regulation was induced by anergic T cells in an Ag-specific manner. Importantly, we found that the T cell-activating capacity of APC remained down-regulated after the anergic T cells were removed. This indicated that anergic T cells conditioned the APC in such a way that the APC became tolerogenic itself. The modulated APC could thus function as a temporal bridge for T cell suppression, in analogy with the recent findings of a conditioned APC being a temporal bridge for T cell activation (2). Interestingly, once down-regulation of the T cell-activating capacity of the APC was established, it also affected T cell responses directed to other epitopes presented by the same APC (linked suppression). Moreover, we found that anergic T cells were able to modulate APC previously activated *in vitro* or *in vivo*, demonstrating the dominant nature of the tolerogenic effect mediated by anergic T cells.

[0016] Modulation of the T cell-activating capacity of APC has been reported in several studies. Modulation can be mediated via down-regulation of costimulatory molecules on APC, as was shown in a study using macrophages infected with *Leishmania infantum* parasites (43). In addition, soluble factors such as prostaglandin E_2 or IL-10 can alter the stimulatory capacity of APC (44-47). Modulation of APC was shown furthermore in a study using APC infected with listeriolysin-secreting *Listeria monocytogenes*, which resulted in altered MHC class II/peptide complexes, and the subsequent induction of T cell anergy in responding T cells (48). The active induction of a T cell-inhibitory phenotype in APC by anergic T cells appears to be, however, a sofar not identified immunoregulatory pathway. In our experiments, the reduced T cell-activating capacity of APC pre-cultured with anergic T cells was not associated with selective up- or down-regulation of MHC class II, B7-1/B7-2, or ICAM-1/LFA-1. Furthermore, APC modulation was not mediated via Th2 cells or IL-10/TGFβ producing cells as proposed in other cases (10-12). We have shown previously that the suppressive effect of anergic T cells was not mediated by inhibitory soluble factors, but instead was dependent on cell-cell contact between anergic T cells and APC (19). Interestingly, as anergic T cells were able to modulate APC previously activated *in vitro* or *in vivo,* APC modulation was probably not due to the absence of a molecule on anergic T cells that is involved in APC activation (e.g. CD40L, OX40), but instead, anergic T cells will express a certain surface molecule which upon interaction with its ligand on APC, turns off the APC. The ligand will be present on all APC, as we found that down-regulation of the T cell-activating capacity of APC occurred irrespective of the APC source used.

[0017] It is concluded that whereas helper CD4+ T cells serve to initiate an immune response (49), anergic CD4+ T cells function to control an ongoing immune response via modulation of the APC. As anergic CD4+ T cells are part of the normal peripheral T cell pool, they provide a useful source of immunoregulatory T cells. Recently, Shevach and co-workers demonstrated the existence of a unique population of immunoregulatory CD4+CD25+ T cells (15, 16). The T cells described in their reports share remarkable phenotypic and functional similarities with the anergic T cells used in our experiments, and might therefore represent the naturally occurring anergic T cell population with immunosuppressive capacities. The specificity of the modulatory effect is secured by the fact

that anergic T cells only modulate APC which present their cognate ligand, but importantly, T cell responses directed to additional epitopes presented by the same APC are affected as well. Via this latter phenomenon of linked suppression, the modulated APC might be involved in the induction of anergy in the responding T cells, resulting in infectious tolerance (50, 51). Fine-tuning of the modulatory effects seems to occur as the suppressive capacity of anergic T cells was found to depend on their level of T cell anergy (52, 53).

[0018] As such, anergic T cells should be regarded not only as tolerised cells, but also as active tolerising cells through their capacity to induce APC with tolerogenic function. The Ag-specific induction of anergic T cells may offer therefore a profitable means to reinforce tolerance in situations of chronic inflammation, such as occur during autoimmunity, allergy or transplant rejection.

[0019] Anergic T cells are active mediators of T cell suppression (19). In co-culture experiments, anergic T cells derived from established rat T cell clones and rendered anergic via T cell presentation of the specific antigen (Ag), were active inhibitors of T cell responses. Anergic T cells inhibited not only the responses of T cells with the same antigen specificity as the anergic T cells, but were also capable of efficiently inhibiting polyclonal T cell responses directed to other epitopes. This suppression required cell-cell contact between antigen-presenting cells (APC), anergic T cells and responder T cells, and only occurred when the epitope recognised by the anergic T cell was present. The suppression was not caused by passive competition for ligands on the APC surface, IL-2 consumption, or cytolysis, and was not mediated by soluble factors derived from anergic T cells that were stimulated with their specific Ag. When responder T cells were added 24 hours after co-culturing anergic cells in the presence of Ag and APC, T cell responses were still suppressed, indicating that the suppressive effect was persistently present. Importantly, APC previously capable of stimulating T cells were tolerogenic after incubation in the presence of anergic T cells.

[0020] We compared the effects of Ag presentation by T cells and professional APC on T cell proliferation, cytokine production and surface molecule expression (54). Ag presentation by T cells (T-T presentation) and by professional APC (APC-T presentation) induced an initial T cell activation phase as measured by proliferation and IL-2 production. However, during T-T presentation these responses were lower as compared to Ag presented by APC. In contrast to APC-T presentation, T-T presentation resulted in T cell anergy, as shown by a failure to proliferate or produce IL-2 or IFN upon restimulation with Ag presented by APC. The induction of T cell anergy could not be prevented by addition of rIL-2, indicating that anergy was not due to TCR ligation in the absence of sufficient IL-2. Furthermore, T cells prior activated via T-T presentation did proliferate and produce cytokines upon restimulation with mitogenic stimuli in the presence of APC, indicating that these T cells were not intrinsically defective in their signal transduction pathways. FACS analysis did not reveal differential effects of T-T presentation and APC-T presentation with respect to the expression of CD2, CD4, CD25, CD28, MHC class I and II, and adhesion molecules on activated T cells. In contrast, TCR and CD3 were down-regulated more profoundly during T-T presentation than during APC-T presentation. Moreover, no up-regulation of CD80, CD86, CD45RC and OX40 (CD134) was observed during T-T presentation, whereas increased expression of these molecules was observed during APC-T presentation. Interestingly,

upon antigenic restimulation in the presence of APC, T cells prior activated via T-T presentation still failed to up-regulate these cell surface molecules. The impaired expression of costimulatory and activation molecules (e.g. CD80, CD86, CD45RC and OX40) on T cells after T-T presentation of Ag might therefore lead to altered interactions between T cells and APC upon antigenic restimulation. We propose that T cell anergy is a functional consequence of these altered T cell-APC interactions.

[0021] T cell anergy has been proposed as one of the mechanisms underlying peripheral T cell tolerance. In recent years, the functional relevance of T cell anergy has been studied extensively *in vitro* and *in vivo*, using different species, cell systems and ways to induce anergy. Although these studies concurred about the induction of unresponsiveness, conflicting findings were obtained with respect to the function of anergic T cells, and to the persistence of T cell anergy. We have studied T cell anergy induced through T-T presentation of the specific antigen by rat MHCII+ T cells in the absence of professional APC (53). We have shown show that, depending on the Ag dose with which T cells were incubated, distinct anergic phenotypes were induced. Incubation of T cell clones with a low (suboptimal) Ag dose induced hyporesponsiveness. Incubation with a higher (optimal) Ag dose induced an anergic state capable of exerting immunoregulatory effects. Incubation with a high (supraoptimal) Ag dose led to an anergic suppressive phenotype that was persistent and was not reversed by APC, Ag and rIL-2. These findings demonstrate that T cell anergy is not confined to a single state of functional inactivation. Instead, multiple levels of T cell anergy exist. Anergic T cells can thus contribute to regulation of the immune response either in a persistent and active manner, or in a passive manner, depending on their level of T cell anergy.

EXAMPLES

EXAMPLE 1

Materials and methods

[0022] Rats, T cells and Ag

7-10 week old male inbred Lewis rats ($RT1.^{L}$) were obtained from the University of Limburg (Maastricht, the Netherlands). The isolation, maintenance and properties of the CD4+ T cell clones A2b (25) and Z1a (26) have been described previously. In brief, T cell clone A2b was derived from the draining inguinal lymph nodes (LN) of a Lewis rat immunised with *Mycobacterium tuberculosis* (Mt) in IFA in the base of the tail. A2b is specific for the mycobacterial 65 kD heat shock protein (HSP65) peptide 180-188 (27), and its longer variant peptide 176-190 (28), both of which bind to the Lewis rat MHC class II molecule $RT1.B^{L}$. T cell clone Z1a was derived from the draining popliteal LN of a Lewis rat immunised with myelin basic protein (MBP) in CFA in the hind footpads. Z1a recognises peptide MBP72-85 and the peptide analogue $MBP72\text{-}85_{S79A}$ (S79A), which has a higher $RT1.B^{L}$ binding affinity than the native peptide (29).

[0023] In vitro culture of APC and anergic T cells

Anergy of A2b or Z1a was induced through T-T presentation as described previously (19). In brief, A2b or Z1a T cells ($3x10^{6}$ cells/ml) were incubated with their respective stimulatory peptides 176-190 (10 μg/ml) or S79A (50 μg/ml) in the absence of professional APC. After overnight culture, viable T cells were collected by Ficoll-Isopaque gradient centrifugation, and maintained in culture medium (Iscove's Modified Dulbecco's Medium (IMDM) supplemented with L-Glutamine (2

mM), β-mercaptoethanol (50 μM), penicillin (50 U/ml), streptomycin (50 U/ml) and 2% heat-inactivated normal rat serum, or 10% heat-inactivated FCS (PAA Laboratories)) in the absence of exogenous rIL-2. Anergic T cells were added to APC cultures 3 to 7 days after anergy induction. For APC, splenocytes were used, derived from naive specific pathogen free (SPF) Lewis rats after Ficoll-Isopaque gradient centrifugation. Splenocytes ($1.2x10^7$ cells/ml) were pulsed with peptide 176-190 (10 μg/ml) or S79A (50 μg/ml) for 1-2 hours at 37°C. Peptide-pulsed splenocytes ($2x10^6$/ml) were cultured with non-anergic or anergic T cells ($4x10^5$/ml) at a 5 : 1 ratio in 5-ml cultures in 6 wells plates (Costar) for 16-20 hours in culture medium. In some experiments, splenocytes were cultured with both non-anergic and anergic T cells at a 5 : 1 : 1.5 ratio, added either simultaneously, or anergic T cells were added 6 hours after non-anergic T cells.

[0024] T cell depletion and testing of the T cell-activating capacity of APC

M-450 goat-anti-mouse IgG Dynabeads (Dynal) in PBS ($2x10^8$/ml) were coated overnight at 4°C with 0.1 mg/ml purified R73 (anti-TCRαβ mAb) (30) and 0.5 v/v of OX34 (anti-CD2 mAb) (31) hybridoma cell culture supernatant in PBS + 1% rat serum. For T cell depletion, APC/T cell cultures were incubated with mAb-coated beads (5 beads per target) for 30-45' at 4°C while rolling, followed by magnetic depletion. Depletion of T cell clones A2b and Z1a was confirmed by FACS analysis. No differences in recovery, composition, or viability of isolated APC were observed between splenocytes pre-cultured with non-anergic or anergic T cells. Isolated APC ($1x10^5$ per well) were added in triplicate cultures to responder T cells ($2x10^4$ per well) in flat-bottomed 96 wells plates in culture medium without or with additional Ag, and proliferative responses were assessed by [^{3}H]thymidine incorporation during the last 18 hours of a 96 hours assay. Alternatively, after overnight APC/T cell culture, non-adherent cells were removed by gentle resuspension. Adherent APC were washed twice with IMDM (37°C), by incubating the cells for 30' at 37°C, followed by removal of the medium. A2b responder cells were added at different concentrations to the adherent cells in culture medium. After 3 days, non-adherent (A2b) cells were collected, transferred to 96-wells plates and [^{3}H]thymidine was added for 16 hours. For polyclonal *ex vivo* responses, inguinal LN cells (ILNC) were isolated 14 days after immunisation of Lewis rats with 100 μl Mt in IFA (10 mg/ml) in the base of the tail. Popliteal LN cells (PLNC) were isolated 10 days after immunisation of Lewis rats with 50 μl of a 1:1 emulsion of MBP72-85 (1 mg/ml) in CFA (Mt, 4 mg/ml) in each hind footpad. *Ex vivo* polyclonal responses were measured by culturing LNC ($2x10^5$ per well) with Mt (10 μg/ml) or MBP72-85 (10 μg/ml) in the absence or presence of non-anergic or anergic T cells ($2x10^4$ or $6x10^4$ per well).

[0025] Flow cytometry

Depletion of T cell clones A2b and Z1a was confirmed by FACS analysis, by staining with mAb R73 and OX34. T cell clones A2b and Z1a are $R73^{high}$ and $OX34^{high}$, and upon T cell depletion this population was absent. For characterisation of the isolated APC population the following mouse-anti-rat mAb were used: OX33 (IgG1, B cells) (32), OX41 (IgG2a, macrophages/DC) (33) and OX62 (IgG1, DC) (34). Upon depletion using magnetic beads, the majority of the APC population (>90%) consisted of OX33+ cells (B cells). Expression of surface molecules on isolated APC was investigated using the following mouse-anti-rat mAb: OX-6 and OX-17 (anti-MHC class II molecules $RT1.B^L$ and $RT1.D^L$, IgG1) (35), 3H5 and 24F (anti-B7-1 and B7-2, IgG1, kind gifts from Dr. H. Yagita), 1A29 (anti-ICAM-1, IgG1, Pharmingen), and WT.1 (anti-LFA-1, IgG2a,

Pharmingen). Isotype controls were UD15 (anti-chloramphenicol, IgG1) (36) and 1E7 (anti-ovine IgE, IgG2a) (37). The FACS labelling procedure was performed by incubating cells for 30' at 4°C with the appropriate amount of mAb diluted in FACS buffer (PBS, 1% BSA, 0.1% NaN_3, 4% rat serum), followed by two rounds of washing. Cells were incubated with FITC-conjugated goat-anti-mouse secondary Ab (Becton Dickinson) for 30' at 4°C, washed twice, and analysed on a FACScan (Becton Dickinson) using the FL1 channel.

RESULTS

[0026] Anergic T cells specifically down-regulate the T cell-activating capacity of APC

Anergy was induced in the rat CD4+ T cell clones A2b and Z1a through T-T presentation, as described previously (19). In brief, T cells ($3x10^6$/ml) were incubated overnight with their respective stimulatory Ags, the mycobacterial HSP65 peptide 176-190 (10 μg/ml) or MBP72-85 peptide analog S79A (50 μg/ml) in the absence of added professional APC (T-T presentation). Viable T cells were collected, rested, and 3 to 7 days after anergy induction, anergic T cells were added to peptide-pulsed splenocytes derived from naive Lewis rats. As control, splenocytes were cultured with non-anergic T cells. After overnight culture T cells were depleted using magnetic beads coated with mAbs R73 and OX34, directed to TCRαβ and CD2 respectively. T cell depletion was confirmed by FACS analysis. T cell clones A2b and Z1a are $R73^{high}$ and $OX34^{high}$ and these populations were absent upon T cell depletion (Fig. 1A, B). The majority of the isolated APC were OX33+ B cells (>90%, Fig. 1C). No differences in cell composition, recovery and viability of the T cell-depleted APC population were observed after pre-culture with non-anergic or anergic T cells (data not shown). To investigate the T cell-activating capacity of pre-cultured APC, isolated APC ($1x10^5$/well) were cultured with responder T cells ($2x10^4$/well), in the absence or presence of additional Ag, and [^{3}H]thymidine incorporation was measured after 4 days.

[0027] Following the above described protocol, peptide 176-190-pulsed splenocytes were pre-cultured overnight with anergic A2b cells. As control, splenocytes were pre-cultured with non-anergic A2b cells, non-anergic Z1a cells or anergic Z1a cells. After overnight culture, T cells were depleted, and the isolated APC populations were cultured with A2b cells as responder cells. The T cell-activating capacity of APC was assessed in a standard lymphocyte proliferation assay. Fig. 2A shows that the T cell-activating capacity of APC pre-cultured with anergic A2b cells was reduced with >50%, as compared to APC pre-cultured with non-anergic A2b. The APC modulation was Ag-specific, as anergic Z1a cells, specific for peptide S79A, did not modulate APC pulsed with peptide 176-190 (Fig. 2B). Importantly, the T cell-activating capacity of 176-190-pulsed APC pre-cultured with (non) anergic Z1a cells or non-anergic A2b cells was comparable, indicating that the observed difference between non-anergic and anergic A2b cells was not due to up-regulation of the T cell-activating capacity of APC by non-anergic A2b cells. To circumvent the possibility that the APC pre-cultured with anergic A2b cells had internalised or shedded pre-formed $RT1.B^L$/176-190 complexes from the surface, exogenous peptide 176-190 was added during the proliferation assay. The addition of peptide, however, did not overcome the stimulatory deficiency (Fig. 2A). In this experiment, the addition of exogenous peptide did not strongly increase the response of A2b responder cells cultured with the control APC, due to the fact that the maximum proliferative A2b response was already reached with the peptide-pulsed APC (Fig. 2A, black bars). APC were not irradiated after depletion and before use in the proliferation assay. The use of irradiated APC,

however, yielded similar results (data not shown).

[0028] The expression of MHC, costimulatory and adhesion molecules on isolated APC was investigated by FACS analysis in 5 independent experiments. No correlation was found between the specific down-regulation of the T cell-activating capacity of APC by anergic T cells and up- or down-regulated expression on APC of molecules involved in antigen-presentation (MHC I, II), costimulation (B7-1, B7-2) or adhesion (LFA-1, ICAM-1) (data not shown).

[0029] Anergic T cells induce linked suppression via down-regulation of the T cell-activating capacity of APC

Linked suppression has been described as inhibition of T cell proliferation to one Ag leading to inhibition of responses to additional Ags provided that all Ags are presented by the same cell surface (38, 39). It was shown recently that anergic T cells can induce linked suppression (19, 40). To study the mechanism of linked suppression induced by anergic T cells, splenocytes were pulsed with peptide 176-190 or peptide S79A, and cultured overnight with non-anergic or anergic A2b cells, or non-anergic or anergic Z1a cells, respectively. After T cell depletion, APC were added to T cells specific for the ligand that was presented by the APC during pre-culture with anergic T cells (Fig. 3A, 3C), or to T cells specific for a different peptide, which was added to the cultures during the lymphocyte proliferation assay (Fig. 3B, 3D). In all cases, the stimulatory capacity of APC pre-cultured with anergic T cells was diminished during the second culture with either A2b or Z1a T cells in the presence of their specific Ag. This showed that anergic T cells can induce linked suppression as shown in Fig. 3B and 3D, by modulating the stimulatory capacity of APC.

[0030] Anergic T cells down-regulate the T cell-activating capacity of in vitro pre-activated APC

Recent studies have shown that helper T cells can activate APC through CD40L-CD40 interactions to stimulate cytotoxic T cells (2-4). As no reagents against rat CD40 or CD40L are available so far, we pre-cultured peptide 176-190-pulsed APC with non-anergic A2b cells, and we added anergic A2b cells either simultaneously (t=0), or after 6 hours (t=6). As has been described previously CD40L is expressed optimally at 6 hours after T cell activation (41, 42), and this period is sufficient to activate APC via CD40L-CD40 interactions (42). After overnight culture, T cells were depleted and the T cell-activating capacity of APC was investigated. Fig. 4 shows that when anergic T cells were added 6 hours after non-anergic A2b T cells, the anergic cells were still able to modulate the T cell-activating capacity of APC (t=6, open bars). This indicated that anergic T cells were able to overrule the activation signals previously delivered by the non-anergic (helper) T cells. Fig. 5 shows a control experiment to test the activation state of the APC after 6 hours. A2b cells were cultured with 176-190-pulsed splenocytes, and anergic T cells were added either simultaneously, or after 6 hours. When anergic A2b cells were added simultaneously in culture as responder A2b cells, suppression was observed (Fig. 5, t=0). However, anergic T cells were not able to suppress A2b cells that were already activated by APC for 6 hours (Fig. 5, t=6), probably because these T cells did not interact anymore with the APC. This indicated that the chosen time-span of 6 hours, to allow full activation of the APC, was indeed sufficient.

[0031] Anergic T cells down-regulate the T cell-activating capacity of adherent APC

The previous experiments were performed with B cells as APC. We next investigated whether anergic T cells were able to modulate adherent APC, such as macrophages. Peptide 176-190-pulsed

splenocytes were pre-cultured with non-anergic or anergic A2b cells. After overnight culture, non-adherent (T and B) cells were removed by gentle resuspension. Adherent cells were washed twice with Iscove's medium (37°C), and A2b responder cells were added at different concentrations to the adherent APC. After 3 days of culture, A2b T cells were collected and the proliferative response was assessed by [^{3}H]thymidine incorporation. In line with our findings described for B cells, we found that adherent APC pre-cultured with anergic T cells were strongly reduced in their T cell-activating capacity as compared to adherent APC pre-cultured with non-anergic A2b cells (Table 1).

Table 1. Anergic T cells down-regulate the T cell-activating capacity of adherent APC.

Pre-culture with[*]:	non-anergic A2b	anergic A2b
Proliferative A2b response (cpm SD)[†]		
1x10^5 A2b/ml	48 ± 0	18 ± 0
2x10^5 A2b/ml	2,676 ± 384	235 ± 88
4x10^5 A2b/ml	67,929 ± 3,375	480 ± 105
8x10^5 A2b/ml	32,802 ± 1,688	425 ± 136

[*] Splenocytes were pulsed with peptide 176-190, and cultured with non-anergic or anergic A2b cells in 6 wells plates, as described in materials and methods. After overnight culture, non-adherent cells were removed. Adherent cells were washed twice with Iscove=s medium (37°C), and A2b responder cells were added at the indicated concentrations.

[†] After 3 days of culture, A2b T cells were collected, and the proliferative response was measured by [^{3}H]thymidine incorporation for 16 hours.

[0032] Anergic T cells down-regulate the T cell-activating capacity of in vivo activated APC

Finally, we investigated whether anergic T cells were able to down-regulate *in vivo* activated APC. Lewis rats were immunised with *Mycobacterium tuberculosis* (Mt) in IFA. After 14 days, the draining inguinal lymph nodes were removed, and the T cell-activating capacity of *in vivo* activated APC was investigated either in the absence or presence of anergic A2b cells. Fig. 6A shows that the capacity of *in vivo* activated APC to stimulate polyclonal responses to Mt was dramatically reduced in the presence of anergic A2b cells. Similar results were found when Lewis rats were immunised with MBP72-85 in CFA. The T cell-activating capacity of *in vivo* activated APC to stimulate poly-clonal responses to MBP72-85 was completely abrogated by addition of anergic Z1a cells (Fig. 6B).

EXAMPLE 2

[0033] In vivo experiment: The disease modulatory potential of tolerogenic APC is analysed by performing the following experiments in the adjuvant arthritis and experimental autoimmune encephalomyelitis models:

Ex vivo induction of tolerogenic APC:

1) Naïve Lewis rat APC (B cells, macrophages, dendritic cells) are isolated from spleen, blood, or lymph-nodes and pulsed (1x10^7 cells/ml) for 1-4 hours at 37°C with synthetic peptides e.g. MBP72-85, hsp65 176-190, in the absence or presence of other arthritis- or encephalomyelitis-associated antigens.

2) After washing away the excess of unbound peptides, antigen pulsed APC are incubated overnight at 37°C with (non)anergic Z1a or A2b cells (4x10^5 cells/ml) at a ratio of 5:1 in 5-ml cultures in 6-well plates. (The encephalitogenic T cell clone Z1a is specific for MBP72-85, while

the arthritogenic T cell clone A2b is specific for hsp60 176-190).

3) APC are recovered from the culture by depletion of T cells with dynabeads (5 beads per T cell) coated with anti-TCR monoclonal R73 and anti-CD2 monoclonal OX34. APC-T cell cultures are incubated with dynabeads for 30-45 minutes at 4°C while rolling, and T cells are removed via magnetic depletion

4) Tolerogenic APC ($0.02 - 2x10^7$ / 200 µl) are injected intravenously in Lewis rats immunized with Mycobacterium tuberculosis (Mt)/ IFA (5mg/ml Mt in IFA), intracutanously at the base of the tail, to induce adjuvant arthritis (AA), or in Lewis rats immunized subcutanously in the hindpaw with myelin basic protein (MBP) or peptide MBP72-85 in CFA to induce experimental autoimmune encephalomyelitis (EAE). In each experimental group n=5 rats are present. The following groups are compared:

In EAE:

i) APC prepulsed with MBP72-85 and incubated with non-anergic Z1a

ii) APC prepulsed with MBP72-85 and incubated with anergic Z1a

iii) APC prepulsed with hsp60 176-190 with anergic Z1a

iv) APC prepulsed with hsp60 176-190 with anergic A2b

v) APC prepulsed with MBP72-85 + other EAE-associated, e.g. MBP or PLP (proteolipid protein), epitopes recognised in Lewis rats and incubated with anergic Z1a

vi) APC prepulsed with MBP72-85 + hsp epitopes recognised during inflammation in Lewis rats (e.g. epitopes as described in WO 95/25744) and incubated with anergic Z1a

vii) Idem v) but incubated with non-anergic Z1a

viii) Idem vi) but incubated with non-anergic Z1a

In AA:

ix) APC prepulsed with hsp60 176-190 and incubated with non-anergic A2b

x) APC prepulsed with hsp60 176-190 and incubated with anergic A2b

xi) APC prepulsed with MBP72-85 with anergic A2b

xii) APC prepulsed with MBP72-85 with anergic Z1a

xiii) APC prepulsed with hsp60 176-190 + other arthritis-associated epitopes recognised in AA in Lewis rats (e.g. epitopes according to PCT/NL99/00189) and incubated with anergic A2b

xiv) APC prepulsed with hsp60 176-190 + other hsp epitopes recognised during inflammation in Lewis rats (e.g. epitopes according to WO95/25744) and incubated with anergic A2b cells

xv) Idem xiii) but incubated with non-anergic A2b

xvi) Idem xiv) but incubated with non-anergic A2b

5) Disease activity are scored in a blinded setup. For EAE the disease activity are graded from 0-5: 0, no signs; 0.5, weight loss (>5g); 1, limp tail; 2, hind leg weakness; 3, paraplegia; 4, paraplegia with fore limp weakness, moribund condition. Disease activity in the AA model is scored on a scale 0-16. The severity of arthritis is scored by grading each paw from 0-4 based on erythema, swelling, and deformity of the joints and evaluating the weight loss.

Tolerogenic APC are injected at days: 0 (Day of disease induction), 7 (After disease induction, but before clinical signs), 10 in EAE model (Onset of clinical signs) or 14 in AA (Onset of clinical

signs), or at day 20 in AA (During the course of arthritis). These experiments give insights into the specificity of the immunotherapy with tolerogenic APC and into the capacity of such cells to prevent disease induction and to interfere in ongoing disease processes.

EXAMPLE 3

[0034] In vivo application of tolerogenic APC:

We found that anergic T cells can suppress T cells recognising other T cell epitopes provided both antigens are presented by the same APC. The key player in this phenomenon is the tolerogenic APC. This finding implies that in vitro induction of tolerogenic APC can be achieved by using an anergic T cell clone specific for a standard or artificial T cell epitope, and that subsequent loading of the APC with peptides relevant for T cell epitopes involved in the disease processes ensures the specificity of the immunotherapy. The following experiments apply to the two disease models:

In EAE:

i) Transfer of APC loaded with hsp60 176-190 and pre-incubated with anergic A2b

ii) Transfer of APC loaded with hsp60 176-190 + MBP72-85 and pre-incubated with anergic A2b

iii) Transfer of APC loaded with MBP72-85 and pre-incubated with anergic A2b

iv) Transfer of APC loaded with MBP72-85 and pre-incubated with anergic Z1a

In AA:

v) Transfer of APC loaded with MBP72-85 and preincubated with anergic Z1a

vi) Transfer of APC loaded with hsp60 176-190 + MBP72-85 and pre-incubated with anergic Z1a

vii) Transfer of APC loaded with hsp60 176-190 and pre-incubated with anergic Z1a

viii) Transfer of APC loaded with hsp60 176-190 and pre-incubated with anergic A2b

Experiments are performed as described for example 2.

[0035] These data provide evidence for the use of standard T cell clones in vitro for the induction of tolerogenic APC. For immunotherapy of T cell mediated diseases, this would imply that the APC need to be isolated from the blood of the patient. These APC should be loaded ex vivo with the standard peptide and the specific disease-associated peptides, and incubated overnight with the T cell clone specific for the standard peptide. A panel of standard T cell clones is selected for ex vivo induction of tolerogenic APC covering the most frequently used MHC restriction elements. After incubation with anergic T cells the APC are recovered and injected i.v. in saline.

Description of the figures

Figure 1. FACS analysis of the T cell/APC population after T cell depletion using magnetic beads. A2b/splenocyte cultures were stained with anti-TCR mAb R73 (A) and anti-CD2 mAb OX34 (B) before (shaded histograms) and after (open histograms) T cell depletion. $R73^{high}$ $OX34^{high}$ A2b T cells were absent after T cell depletion. (C) Isolated APC were stained with B cell-marker mAb OX33 (open histograms) or with isotype control mAb UD15 (shaded histograms). Similar results were obtained for T cell clone Z1a (data not shown).

Figure 2. Anergic T cells down-regulate the T cell-activating capacity of APC in an Ag-dependent manner. Peptide 176-190-pulsed splenocytes were cultured overnight with non-anergic (solid bars) or anergic (hatched bars) A2b (A) or Z1a cells (B). After overnight culture, T cells were depleted, and the T-cell activating capacity of APC was investigated in a lymphocyte proliferation assay using A2b cells as responder T cells. On the x-axis the amount of exogenous peptide added during the lymphocyte proliferation assay is indicated. One representative experiment out of 4 independent experiments is shown.

Figure 3 Anergic T cells induce linked suppression via down-regulation of the T cell-activating capacity of APC. (A, B) Peptide 176-190-pulsed splenocytes were cultured overnight with non-anergic (solid bars) or anergic (hatched bars) A2b cells. (C, D) Peptide S79A-pulsed splenocytes were cultured overnight with non-anergic (solid bars) or anergic (hatched bars) Z1a cells. After T cell depletion, the T cell-activating capacity of APC was investigated using A2b cells (A, D) or Z1a cells (B, C) as responder T cells, in the absence (med) or presence of exogenously added peptide. The data are representative for 2 independent experiments for each T cell clone.

Figure 4. Anergic T cells down-regulate the T cell-activating capacity of *in vitro* activated APC. (A) Peptide 176-190-pulsed splenocytes were cultured with non-anergic (solid bars) or anergic (hatched bars) A2b cells, or with a mix of non-anergic:anergic A2b cells (1 : 1.5, open bars). In mixed cultures, anergic T cells were added simultaneously with non-anergic T cells (t=0) or 6 hours later (t=6). After overnight culture, T cells were depleted, and the T cell-activating capacity of APC was investigated using A2b cells as responder cells.

Figure 5. Peptide 176-190-pulsed splenocytes were cultured with A2b cells in the absence (1 : 0, solid bars) or presence (1 : 1.5, hatched bars) of anergic A2b cells. Anergic T cells were added simultaneously with non-anergic T cells (t=0), or 6 hours later (t=6). Proliferative responses of these mixed cultures were measured after 4 days. One out of 2 independent experiments is shown.

Figure 6. Anergic T cells down-regulate the T-cell activating capacity of *in vivo* activated APC. (A) Lewis rats were immunised in the base of the tail with Mt/IFA. *Ex vivo* (day 14) draining ILNC responses to Mt (10 g/ml) were measured in the absence (solid bars) or presence (1 : 0.1, open bars; 1 : 0.3 hatched bars) of (non) anergic A2b cells. (B) Lewis rats were immunised with MBP72-85 in CFA in each hind footpad. *Ex vivo* (day 10) draining PLNC responses to MBP72-85 (10 g/ml) were measured in the absence (solid bars) or presence (1 : 0.1, open bars; 1 : 0.3 hatched bars) of (non) anergic Z1a cells. Results are representative for 1 out of 9 different Mt/IFA-immunised animals, and 1 out of 3 different MBP72-85/CFA-immunised animals.

REFERENCES

1 **Mitchison, N. A. and O'Malley, C.**, Three-cell-type clusters of T cells with antigen-presenting cells best explain the epitope linkage and noncognate requirements of the in vivo cytolytic response. *Eur. J. Immunol.* **17**: 1579 (1987).

2 **Ridge, J. P., Di Rosa, F. and Matzinger, P.**, A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* **393**: 474 (1998).

3 **Schoenberger, S. P., Toes, R. E. M., van der Voort, E. I. H., Offringa, R. and Melief, C. J. M.**, T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* **393**: 480 (1998).

4 **Bennett, S. R. M., Carbone, F. R., Karamalis, F., Flavell, R. A., Miller, J. F. A. P. and Heath, W. R.**, Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* **393**: 478 (1998).

5 **Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A. and Alber, G.**, Ligation

of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J. Exp. Med.* **184:** 747 (1996).

6 **D'Orazio, T. J. and Niederkorn, J. Y.,** A novel role for TGF-β and IL-10 in the induction of immune privilege. *J. Immunol.* **160:** 2089 (1998).

7 **Brunner, M. C., Mitchison, N. A. and Schneider, S. C.,** Immunoregulation mediated by T cell clusters. *Folia Biologica (Praha)* **40:** 359 (1994).

8 **Lafaille, J. J., Nagashima, K., Katsuki, M. and Tonegawa, S.,** High incidence of spontaneous autoimmune encephalomyelitis in immunodeficient anti-myelin basic protein T cell receptor transgenic mice. *Cell* **78:** 399 (1994).

9 **Saoudi, A., Seddon, B., Hetah, V., Fowell, D. and Mason, D.,** The physiological role of regulatory T cells in the prevention of autoimmunity: the function of the thymus in the generation of the regultory T cell subset. *Immunol. Rev.* **149:** 195 (1996).

10 **Liblau, R. S., Singer, S. M. and McDevitt, H. O.,** Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. *Immunol. Today* **16:** 34 (1995).

11 **Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., De Vries, J. E. and Roncarolo, M. G.,** A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* **389:** 737 (1997).

12 **Weiner, H. L.,** Oral tolerance: immune mechanisms and treatment of autoimmune diseases. *Immunol. Today* **18:** 335 (1997).

13 **Powrie, F. and Mason, D.,** OX-22 high CD4+ T cells induce wasting disease with multiple organ pathology: prevention by the OX-22 low subset. *J. Exp. Med.* **172:** 1701 (1990).

14 **Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. and Toda, M.,** Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* **155:** 1151 (1995).

15 **Suri-Payer, E., Amar, A. Z., Thornton, A. M. and Shevach, E. M.,** CD4+CD25+ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J. Immunol.* **160:** 1212 (1998).

16 **Thornton, A. M. and Shevach, E. M.,** CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* **188:** 287 (1998).

17 **Lombardi, G., Sidhu, S., Batchelor, R. and Lechler, R.,** Anergic T cells as suppressor cells in vitro. *Science* **264:** 1587 (1994).

18 **Diepolder, H. M., Jung, M.-C., Wierenga, E., Hoffmann, R. M., Zachoval, R., Gerlach, T. J., Scholz, S., Heavner, G., Riethmüller, G. and Pape, G. R.,** Anergic Th1 clones specific for hepatitis B virus (HBV) core peptides are inhibitory to other HBV core-specific CD4+ T cells in vitro. *J. Virol.* **70:** 7540 (1996).

19 **Taams, L. S., van Rensen, A. J. M. L., Poelen, M. C. M., van Els, C. A. C. M., Besseling, A. C., Wagenaar, J. P. A., van Eden, W. and Wauben, M. H. M.,** Anergic T cells actively suppress T cell responses via the antigen presenting cell. *Eur. J. Immunol.* **28:** 2902 (1998).

20 **Rocha, B., Grandien, A. and Freitas, A. A.,** Anergy and exhaustion are independent mechanisms of peripheral tolerance. *J. Exp. Med.* **181:** 993 (1995).

21 **Schwartz, R. H.,** A cell culture model for T lymphocyte clonal anergy. *Science* **248:** 1349 (1990).

22 **Ko, H., Fu, S., Winchester, R. J., Yu, D. T. Y. and Kunkel, H. G.,** Ia determinants on stimulated human T lymphocytes. Occurence on mitogen- and antigen-activated T cells. *J. Exp. Med.* **150:** 246 (1979).

23 **Broeren, C. P. M., Wauben, M. H. M., Lucassen, M. A., van Meurs, M., van Kooten, P. J. S., Boog, C. J. P., Claassen, E. and van Eden, W.,** Activated rat T cells synthesize and express functional major histocompatibility class II antigens. *Immunology* **84:** 193 (1995).

24 **Lamb, J. R., Skidmore, B. J., Green, N., Chiller, J. M. and Feldmann, M.,** Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza hemagglutinin. *J. Exp. Med.* **157:** 1434 (1983).

25 **Holoshitz, J., Matitiau, A. and Cohen, I. R.,** Arthritis induced in rats by cloned T lymphocytes responsive to Mycobacteria but not to collagen type II. *J. Clin. Invest.* **73:** 211 (1984).

26 **Ben-Nun, A., Wekerle, H. and Cohen, I. R.,** The rapid isolation of clonable antigen-specific T lymphocytes lines capable of mediating autoimmune encephalomyelitis. *Eur. J. Immunol.* **11:** 195 (1981).

27 **Van Eden, W., Thole, J. E. R., van der Zee, R., Noordzij, A., van Embden, J. D. A., Hensen, E. J. and Cohen, I. R.,** Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. *Nature* **331:** 171 (1988).

28 **Anderton, S. M., van der Zee, R., Noordzij, A. and van Eden, W.,** Differential mycobacterial 65-kDa heat shock protein T cell epitope recognition after adjuvant arthritis-inducing or protective immunization protocols. *J. Immunol.* **152:** 3656 (1994).

29 **Wauben, M. H. M., Joosten, I., Schlief, A., van der Zee, R., Boog, C. J. P. and van Eden, W.,** Inhibition of experimental autoimmune encephalomyelitis by MHC class II binding competitor peptides depends on the relative MHC binding affinity of the disease-inducing peptide. *J. Immunol.* **152:** 4211 (1994).

30 **Hünig, T., Wallny, H.-J., Hartley, J. K., Lawetzky, A. and Tiefenthaler, G.,** A monoclonal antibody to a constant determinant of rat T cell antigen receptor that induces T cell activation. *J. Exp. med.* **169:** 73 (1989).

31 **Williams, A. F., Barclay, A. N., Clark, S. J., Paterson, D. J. and Willis, A. C.,** Similarities in sequences and cellular expression between rat CD2 and CD4 antigens. *J. Exp. Med.* **165:** 368 (1987).

32 **Woollett, G. R., Barclay, A. N., Puklavec, M. and Williams, A. F.,** Molecular and antigenic heterogeneity of the rat leukocyte-common antigen from thymocytes and T and B lymphocytes. *Eur. J. Immunol.* **15:** 168 (1985).

33 **Robinson, A. P., White, T. M. and Mason, D. W.,** Macrophage heterogeneity in the rat as dileated by two monoclonal antibodies MRC OX-41 and MRC OX-42, the latter recognizing complement receptor type 3. *Immunology* **57:** 239 (1986).

34 **Brenan, M. and Puklavec, M.,** The MRC OX-62 antigen: a useful marker in the purification of rat veiled cells with the biochemical properties of an integrin. *J. Exp. Med.* **175:** 1457 (1992).

35 **Fukumoto, T., McMaster, W. R. and Williams, A. F.,** Mouse monoclonal antibodies against rat major histocompatibility antigens. Two Ia antigens and expression of Ia and class I antigens in rat thymus. *Eur. J. Immunol.* **12:** 237 (1982).

36 **van de Water, C., Haagsma, N., van Kooten, P. J. S. and van Eden, W.,** An enzyme-linked immunosorbent assay for the determination of chloramphenicol using a monoclonal antibody. *Zeitschrift für Lebensmittel-Untersuchung und Forschung* **185:** 202 (1987).

37 **Kooyman, F. N. J., van Kooten, P. J. S., Huntley, J. F., MacKellar, A., Cornelissen, A. W. C. A. and Schallig, H. D. F. H.,** Production of a monoclonal antibody specific for ovine immunoglobulin E and its application to monitor serum IgE responses to *Haemonchus contortus* infection. *Parasitology* **114:** 395 (1997).

38 **Holán, V. and Mitchison, N. A.,** Haplotype-specific suppressor T cells mediating linked suppression of immune responses elicited by third-party H-2 alloantigens. *Eur. J. Immunol.* **13:** 652 (1983).

39 **Davies, J. D., Leong, L. Y. W., Mellor, A., Cobbold, S. P. and Waldmann, H.,** T cell suppression in transplantation tolerance through linked recognition. *J. Immunol.* **156:** 3602 (1996).

40 **Frasca, L., Carmichael, P., Lechler, R. and Lombardi, G.,** Anergic T cells effect linked suppression. *Eur. J. Immunol.* **27:** 3191 (1997).

41 **Lederman, S., Yellin, M. J., Krichevsky, A., Belko, J., Lee, J. J. and Chess, L.,** Identification of a novel surface protein on activated CD4+ T cells that induces contact-dependent B cell differentiation (help). *J. Exp. Med.* **175:** 1091 (1992).

42 **Wagner, J., D. H., Stout, R. D. and Suttles, J.,** Role of the CD40-CD40 ligand interaction in CD4+ T cell contact-dependent activation of monocyte interleukin-1 synthesis. *Eur. J. Immunol.* **24:** 3148 (1994).

43 **Pinelli, E., Rutten, V. P. M. G., Bruysters, M., Moore, P. F. and Ruitenberg, E. J.,** Compensation for decreased expression of B7 molecules on Leishmania infantum-infected canine macrophages results in restoration of parasite-specific T cell proliferation and IFNγ production. *Infection and Immunity.* In press (1998).

44 **Kalinski, P., Hilkens, C. M. U., Snijders, A., Snijdewint, F. G. M. and Kapsenberg, M. L.,** IL-12-deficient dendritic cells, generated in the presence of prostaglandin E_2, promote type 2 cytokine production in maturing human naive T helper cells. *J. Immunol.* **159:** 28 (1997).

45 **De Waal Malefyt, R., Haanen, J., Spits, H., Roncarolo, M. G., te Velde, A., Figdor, C., Johnson, K., Kastelein, R., Yssel, H. and de Vries, J. E.,** Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by dimisshing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J. Exp. Med.* **174:** 915 (1991).

46 **Willems, F., Marchant, N., Delville, J. P., Gerard, C., Delvaux, A., Velu, T., de Boer, M. and Goldman, M.,** Interleukin-10 inhibits B7 and intercellular adhesion molecule-1 expression on human monocytes. *Eur. J. Immunol.* **24:** 1007 (1994).

47 **de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C. G. and de Vries, J. E.,** Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med.* **174:** 1209 (1991).

48 **Darji, A., Stockinger, B., Wehland, J., Chakraborty, T. and Weiss, S.,** T cell anergy induced by antigen presenting cells treated with the hemolysin of Listeria monocytogenesis. *Immunol. Lett.* **57:** 33 (1997).

49 **Guerder, S. and Matzinger, P.,** A fail-safe mechanism for maintaining self-tolerance. *J. Exp. Med.* **176:**

553 (1992).

50 **Qin, S., Cobbold, S. P., Pope, H., Elliott, J., Kioussis, D., Davies, J. and Waldmann, H.,** "Infectious" transplantation tolerance. *Science* **259:** 974 (1993).

51 **Waldmann, H. and Cobbold, S.,** How do monoclonal antibodies induce tolerance? A role for infectious tolerance? *Annu. Rev. Immunol.* **16:** 619 (1998).

52 **Taams, L. S., Wauben, M. H. M. and van Eden, W.,** Antigen specific immunosuppressive effects of T cells with distinct levels of anergy. *Proceedings of the 10th international congress on Immunology.* New Delhi, India. In press (1998).

53 **Taams, L. S., van Eden, W. and Wauben, M. H. M.,** Dose-dependent induction of distinct anergic phenotypes: Multiple levels of T cell anergy. *J .Immunol.* **162:** 1974 (1999).

54 **Taams, L. S., van Eden, W. and Wauben, M. H. M.,** Antigen presentation by T cells versus professional APC: Differential consequences for T cell activation and subsequent T cell-APC interactions. *Eur. J. Immunol.* **29:** 1543 (1999).

CLAIMS

1. An isolated tolerogenic antigen-presenting cell (APC), the tolerogenicity being induced by treatment of an APC with anergic T cells or active components or analogues thereof, said tolerogenicity being characterised by a) reduced induction of T cell activation upon T-cell receptor ligation and b) a dominant tolerogenic effect.

2. A tolerogenic APC according to claim 1, wherein the reduced induction is such that T-cell response is inhibited by at least 50%.

3. A tolerogenic APC according to claim 1 or 2, wherein the APC is selected from B cells, macrophages and dendrites, in particular from B cells and macrophages.

4. An artificial cell in the form of a liposome having the immunologic characteristics of the tolerogenic APC according to any one of claims 1-3.

5. A pharmaceutical composition comprising a tolerogenic antigen presenting cell according to any of the claims 1-3 or an artificial cell accoording to claim 4, as a therapeutically active component in combination with a pharmaceutically acceptable carrier.

6. A pharmaceutical composition according to claim 5, in a medicinal dosage form, in particular in the form of an isotonic solution suitable for injection.

7. A pharmaceutical composition according to claim 5 or 6, further comprising a therapeutically active peptide or protein.

8. A pharmaceutical composition according to claim 7, wherein said therapeutically active peptide or protein is selected from auto-antigens, allo-antigens and allergens.

9. A pharmaceutical composition according to claim 8, wherein said therapeutically active peptide or protein is selected from myelin basic protein (MBP), myelin oligodendrocyte-associated protein (MOG), collagen, heat-shock proteins, arthritis-related peptides or proteins, Der P1, Fel D1, acetyl choline receptor molecules, and Major and Minor Histocompatibility Complex-derived peptides or proteins.

10. Use of a tolerogenic APC according to any one of claims 1-4, for preparing a pharmaceutical composition method for the prophylaxis and/or treatment of auto-immune disease, allergy or allo-graft rejection..

11. A method for inducing T-cell tolerance, comprising administering to a subject requiring such induction a therapeutically effective amount of a pharmaceutical composition according to any one of claims 5-9.

12. A method according to claim 10 or 11, wherein the subject is suffering from any of the diseases rheumatoid arthritis, multiple sclerosis, psoriasis, diabetes, T-cell mediated allergies and allograft rejection.

13. A method according to claim 11 or 12, comprising deriving antigen presenting-cells from said subject, making with tolerogenic using T cells not derived from said subject and rendered anergic by treatment with an antigen not related to the disease to be treated, and administering the tolerogenic cells together with a disease-related protein or peptide.

14. A method for producing tolerogenic antigen-presenting cells (APC), comprising in vitro culture of antigen-presenting cells in the presence of anergic T cells or active components or analogues thereof under tolerogenicity-inducing conditions.

15. A method according to claim 14, wherein said T cells are derived from a T cell line.

16. A method for producing a soluble factor or factors responsible for the inhibition of antigen-specific T-cell response, comprising determining the difference in expression of cytokine between activated antigen presenting cells that. have not undergone the treatment according to claim 14 and tolerogenic antigen presenting cells obtainable according to the method of claim 14 or 15.

17. A method for producing the nucleic acid sequence encoding a factor or factors responsible for the inhibition of antigen specific T-cell response, comprising determining the difference in mRNA expression of soluble factors between activated antigen presenting cells that have not undergone the treatment according to claim 14 and tolerogenic antigen presenting cells obtainable according to the method of claim 14 or 15.

18. A method for producing a component or components responsible for the inhibition of antigen specific T-cell response, comprising determining the difference in expression of cell surface expressed protein between activated antigen presenting cells that have not undergone the treatment according to claim 14 and tolerogenic antigen presenting cells obtainable according to the method of claim 14 or 15.

19. A method for producing the nucleic acid sequence encoding a component or components responsible for the inhibition of antigen specific T-cell response, comprising determining the difference in mRNA expression of cell surface protein between activated antigen presenting cells that have not undergone the treatment according to claim 14 and tolerogenic antigen presenting cells obtainable according to the method of claim 14 or 15.

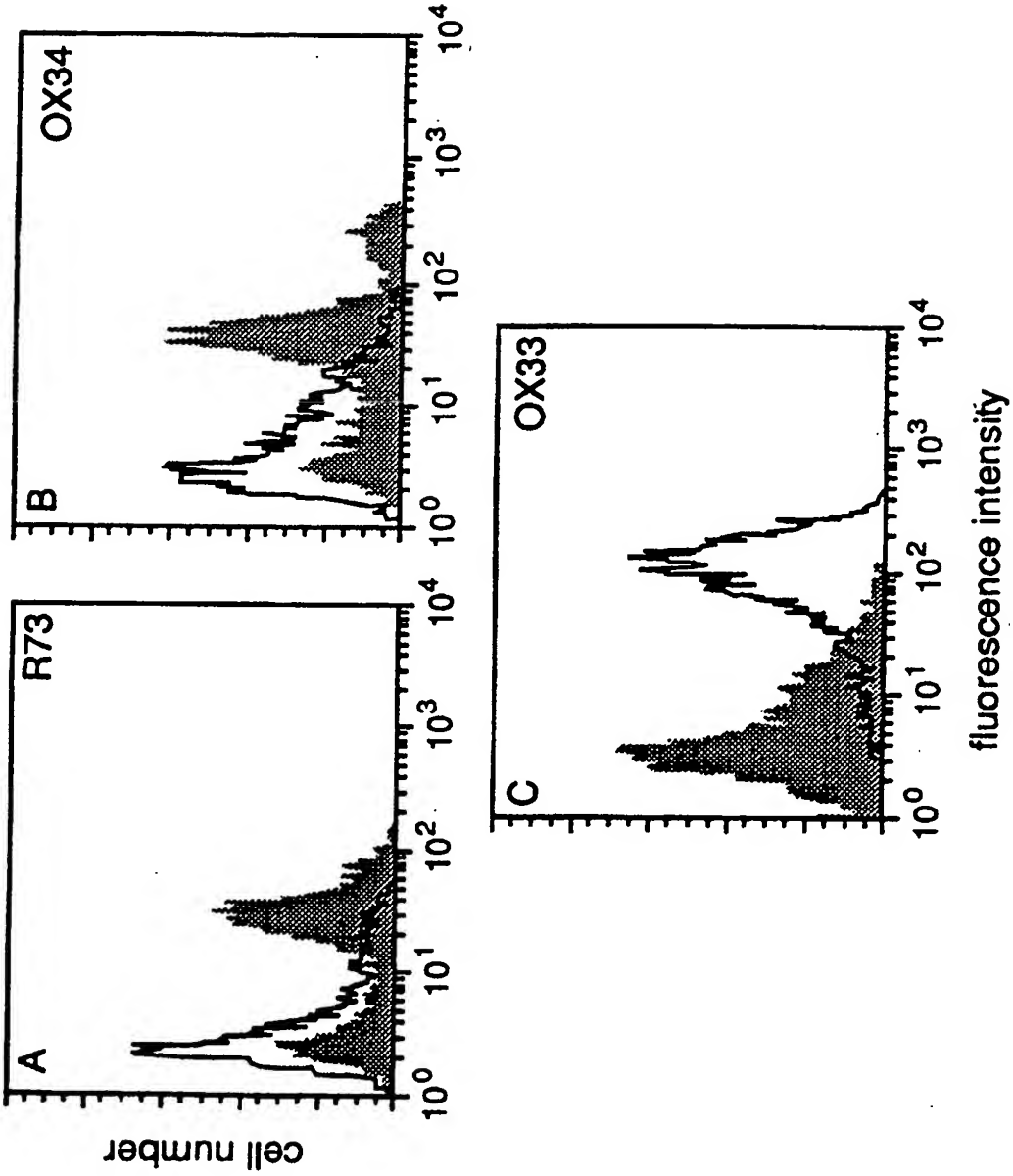

A
R73
B
OX34
C
OX33
cell number
fluorescence intensity
10^0
10^1
10^2
10^3
10^4

Fig 1

Fig 2

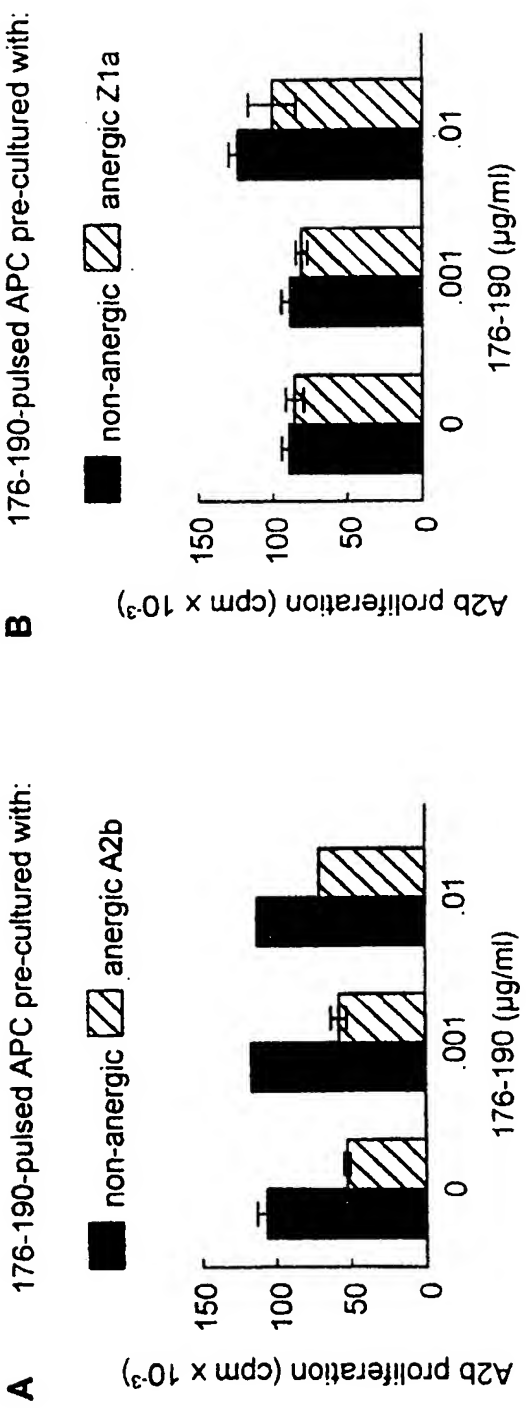

A
176-190-pulsed APC pre-cultured with:
non-anergic
anergic A2b
A2b proliferation (cpm x 10-3)
150
100
50
0
0
.001
.01
176-190 (µg/ml)
B
176-190-pulsed APC pre-cultured with:
non-anergic
anergic Z1a
A2b proliferation (cpm x 10-3)
150
100
50
0
0
.001
.01
176-190 (µg/ml)

Fig 3

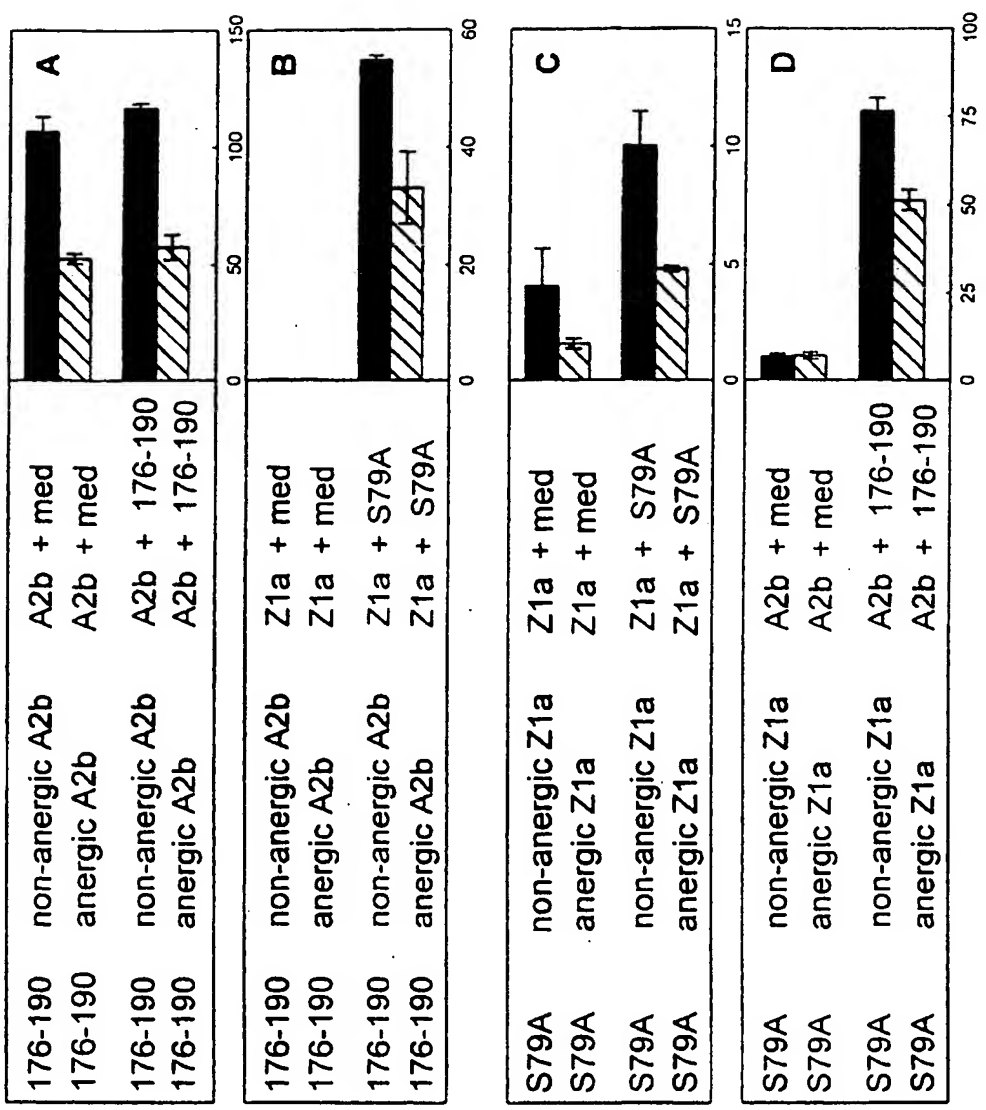

APC pulsed with: pre-cultured with: second culture with: proliferation (cpm x 10-3)
176-190 non-anergic A2b A2b + med
176-190 anergic A2b A2b + med
176-190 non-anergic A2b A2b + 176-190
176-190 anergic A2b A2b + 176-190
A
0 50 100 150
176-190 non-anergic A2b Z1a + med
176-190 anergic A2b Z1a + med
176-190 non-anergic A2b Z1a + S79A
176-190 anergic A2b Z1a + S79A
B
0 20 40 60
S79A non-anergic Z1a Z1a + med
S79A anergic Z1a Z1a + med
S79A non-anergic Z1a Z1a + S79A
S79A anergic Z1a Z1a + S79A
C
0 5 10 15
S79A non-anergic Z1a A2b + med
S79A anergic Z1a A2b + med
S79A non-anergic Z1a A2b + 176-190
S79A anergic Z1a A2b + 176-190
D
0 25 50 75 100

Fig 4

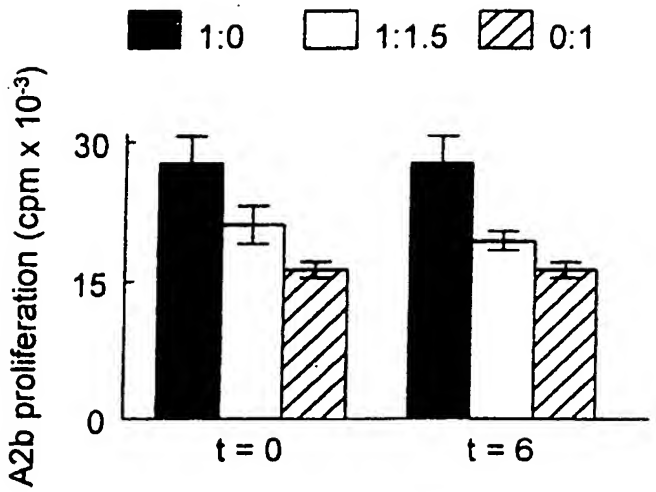

1:0
1:1.5
0:1
A2b proliferation (cpm x 10-3)
30
15
0
t = 0
t = 6

Fig 5

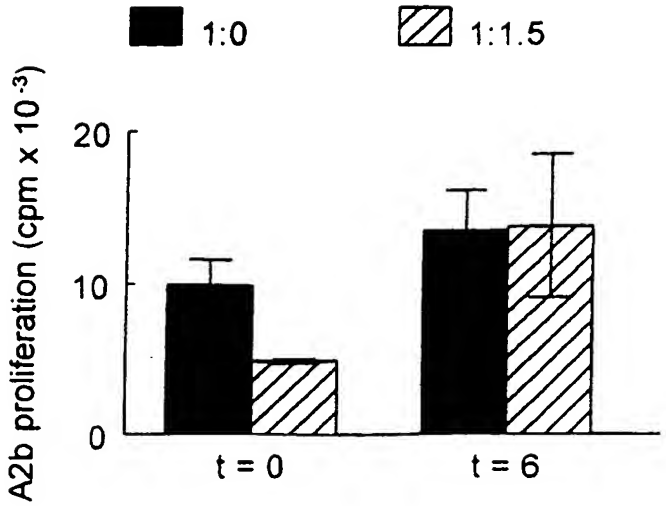
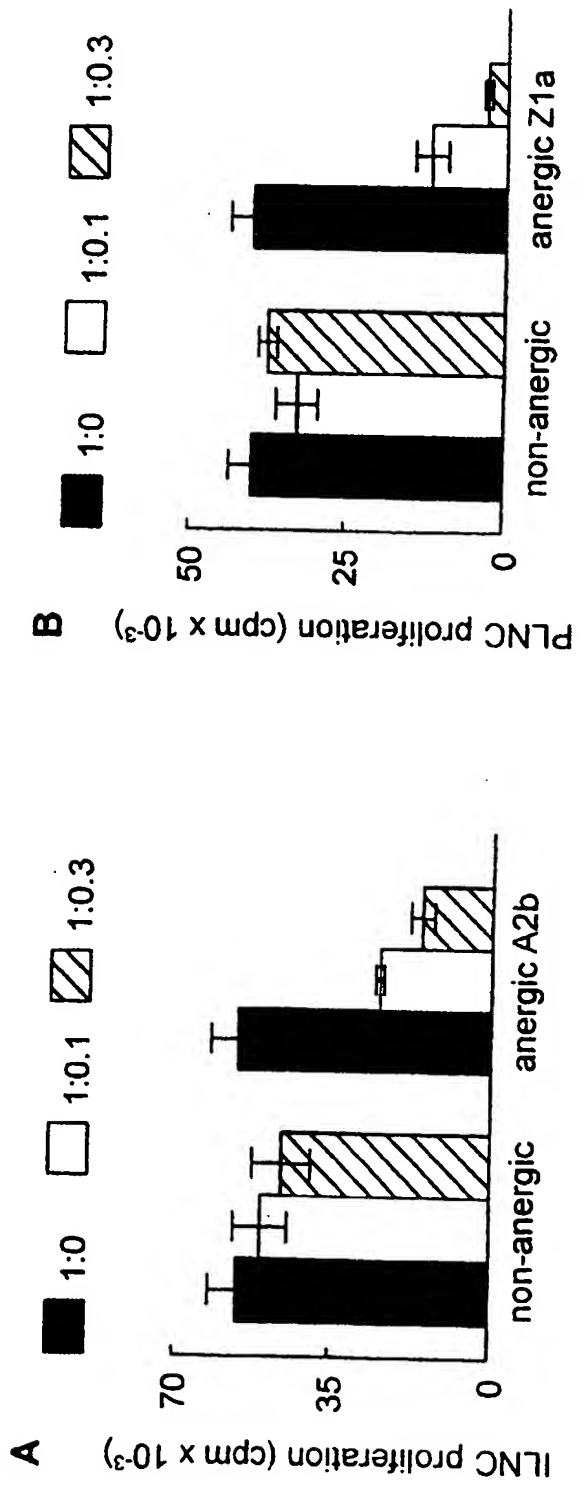

1:0
1:1.5
A2b proliferation (cpm x 10-3)
20
10
0
t = 0
t = 6

Fig 6


A
ILNC proliferation (cpm x 10-3)
70
35
0
1:0
1:0.1
1:0.3
non-anergic
anergic A2b
B
PLNC proliferation (cpm x 10-3)
50
25
0
1:0
1:0.1
1:0.3
non-anergic
anergic Z1a